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THE INFLUENCE OF VERAPAMIL AND NICARDIPINE ON THE RATE OF
METABOLISM OF MIDAZOLAM

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ABSTRACT

Certified Registered Nurse Anesthetists administer numerous drugs to surgical patients who may be on a wide and varied pharmacological regimen. It is important that the nurse anesthetist be aware of potential drug-drug interactions. The purpose of this study is to assess the *in vitro* metabolic reactions of midazolam in the presence of the calcium channel blockers verapamil and nicardipine in hepatic microsomes from three different human livers. Midazolam a widely used premedicant in anesthesia, and the calcium channel blockers verapamil and nicardipine are all metabolized by the cytochrome *P450* 3A4 subfamily of liver microsomal enzymes. Recent clinical observations and experimental studies suggest that midazolam's effects can be augmented by microsomal inhibition when drugs compete for the same family of metabolizing enzymes. In this study the metabolism of midazolam's major metabolite alpha hydroxymidazolam was measured in the presence of differing concentrations of calcium channel blockers using human liver microsomes. Data were analyzed using regression analysis to determine the percent of inhibition and metabolism and Lineweaver-Burk plots were used to determine the inhibition constant (K_i). The mean K_i for nicardipine was 1.35 and 29.3 for verapamil. Nicardipine was the stronger inhibitor of the two calcium channel blockers. Midazolam's effects in the presence of nicardipine and verapamil may be augmented or prolonged.

Key Words: midazolam nicardipine verapamil metabolism inhibition microsomes cytochrome P450

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DEDICATION

To my wife, I dedicate this thesis to you. Your love and support strengthened our life-long bond. You unselfishly took a backseat for the past two years in order for me to fulfill a life's goal. I love you.

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CHAPTER I: INTRODUCTION

Background

Certified Registered Nurse Anesthetists (CRNAs) work in a poly-pharmacologic world. On any given day in any surgery a CRNA can administer up to ten to twenty drugs to an individual patient. In addition, many patients are already taking several medications for various reasons. This is especially true for elderly patients and those with acute or chronic ailments who are being treated with multiple drugs. The incidence of drug interactions increases as the number of drugs a person receives increases. The potential for adverse drug interactions is probably no where else greater than during the administration of anesthesia (Bovill, 1997).

Drug interactions occur because the effect of one drug is altered by the action of another drug. Drug interactions may have synergistic effects as with opioids and hypnotics. However, many drug interactions have deleterious effects on patients. It is important that CRNAs be pharmacologically astute because they administer so many drugs, the “medicine chest” that so often accompanies a patient to surgery increases the potential for adverse drug interactions (Bovill, 1997).

Drugs belonging to the benzodiazepine family are commonly used in anesthesia. In fact, benzodiazepines have replaced barbituates in many situations because of their more favorable pharmacologic actions such as anterograde amnesia, less depression of the respiratory and cardiovascular systems, anticonvulsant actions, relative safety in overdose, and lower incidence of physical dependence (Stoelting, 1991).

Midazolam is a member of the benzodiazepine family. It is a popular drug used as a premedicant and for induction and maintenance of anesthesia. Midazolam produces anxiolysis, amnesia, hypnosis, skeletal muscle relaxation, and anticonvulsant effects. In comparison with other benzodiazepines, midazolam has a quicker onset, shorter duration of action, and greater potency (Dundee, Halliday, Harper, & Brogden, 1984; Stoelting, 1991). When used correctly midazolam is considered to be a safe drug. However, there have been several reports about the enhanced effects of midazolam, and negative outcomes when used in conjunction with other drugs. Gascon and Dayer (1991) noted enhanced effects of midazolam with negative outcomes on a 61 year-old male admitted to the hospital for chest pain and atrial fibrillation. In this report, the patient was noted to have received 400 mg a day of erythromycin for suspected Legionnaire's disease. In addition, he was given 1.7 grams of amiodarone over three days to control his cardiac dysrhythmia. During electrical cardioversions the patient was given 300 mg of intravenous midazolam over 14 hours to achieve sedation. The expected elimination half-life of midazolam is 1.5 to 2 hours, this patient slept for six days. The terminal half-life of midazolam in this patient was reported to be 24.8 hours. The investigators found in in-vitro studies that erythromycin is a selective and powerful inhibitor of cytochrome P450 DB1 and also acts as a competitive inhibitor of the hydroxylation of midazolam.

Unconsciousness has also been reported in an eight-year old child who prior to adeniodectomy was premedicated with 0.5 mg/kg of oral midazolam (27 kg) for sedation. The child was taken to the operating room in a conscious state with stable vital signs. An infusion of 400 mg of erythromycin was begun as prophylaxis for an asymptomatic ventricular septal defect. Ten minutes into the infusion the child became nauseous and

tachycardic. When 200 mg of erythromycin had been infused (40 minutes after beginning the infusion) the child lost consciousness. The child was taken to the recovery room and the case postponed. The child had spontaneous respirations but was unresponsive to noxious stimuli. After 45 minutes in the recovery room the child awoke drowsy but had no other ill effects. Reduced hepatic clearance appeared to be the best explanation for high midazolam concentration. It was thought this could be interindividual variation but more probably the result of enzyme inhibition by erythromycin (Hiller, Olkkola, Isohanni, & Saarnivaara, 1990).

Additional studies have shown that midazolam when given with certain pharmacological therapies in the surgical environment can result in negative outcomes. In a study conducted by Dundee, Collier, Carlisle, and Harper (1986) it was found that the elimination half-life of midazolam was prolonged in 14 of 217 patients. Defective hepatic metabolism of midazolam was thought to be a factor. It should be noted that midazolam half-life was significantly prolonged in patients who underwent major operations and therefore entered a poly-pharmacologic environment. Further effects of midazolam causing prolonged sedation have been reported by (Backman, Olkkola, Arnako, Himberg, & Neuvonen, 1994) when used in combination with the calcium channel blockers verapamil and diltiazem.

Despite possible negative outcomes with midazolam during additional pharmacologic treatment, midazolam is widely used in anesthesia. There are many possible combinations of drugs that can be given with midazolam. Of interest is the effect of the calcium channel blockers (CCBs) verapamil and nicardipine on the rate of metabolism of midazolam.

Calcium plays a fundamental role in myocardial and vascular smooth muscle contraction. Calcium entry into the cell allows calcium in the sarcoplasmic reticulum to be released. Contraction of the heart occurs because of the actin-myosin interactions which are facilitated by the troponin/calcium complex. Calcium plays a similar role in vascular smooth muscle. The exception here is a calmodulin/calcium complex, which allows for the actin-myosin interaction and subsequent contraction of the muscle (Durand, Lehot, and Foex, 1991).

Calcium entry into a cell is accomplished by either voltage operated channels or receptor operated channels. Calcium channel blockers bind to voltage operated channels and significantly decrease their opening. This effectively decreases the current of calcium, which traverses the cell membrane. The result is a decrease in cardiac contractility and a slowing of the sino-atrial and atrio-ventricular pacemakers in the myocardium. Blocking calcium channels in vascular smooth muscle inhibits the tissue's ability to contract. Therefore these drugs exhibit vasodilation, and negative inotropic and chronotropic properties. This in effect lowers the oxygen demand placed upon the heart and dilates the vessels supplying the heart and the muscles of the body (Durand et al., 1991).

Calcium channel blockers have been divided into two groups by the World Health Organization. Group A consists of CCBs that are selective for slow calcium channels. Channels of excitable cells, those able to produce an action potential, have a system of gates for the inward transfer of calcium, sodium, and potassium. Theoretically, slow calcium channels and fast sodium channels are present in these cells. Slow calcium channels have an affinity that is 100 times greater for calcium than for other ions. These

slow channels are numerous in the sino-atrial and atrio-ventricular nodes. Group B consists of those CCBs that do not have a high affinity for the slow channels. Verapamil and nicardipine are members of group A (Durand et al., 1991).

Although CCBs work in the same ways, slowing of heart rate, lessening contractile force, and vasodilation, verapamil and nicardipine are different in respect to structure and major effects. Verapamil is a non-dihydropyridine with strong negative inotropic and chronotropic properties. Nicardipine a dihydropyridine, on the other hand has greater effects upon vasodilation than on coronary rate and force (Katzung, 1998).

Calcium channel blocking drugs are widely used and have documented effectiveness in angina, hypertension, and supraventricular tachycardias. They are also of use in hypertrophic cardiomyopathy, migraine, atherosclerosis, and Raynaud's syndrome (Katzung, 1998). The beneficial effects of decreases in oxygen demand and coronary and peripheral vasodilation increase the chances that individuals with any of the above illnesses might be taking a CCB such as verapamil or nicardipine. Therefore the likelihood of a surgical patient who is routinely on these CCBs would not be uncommon. Thus the potential for drug-drug interactions in anesthesia between midazolam and verapamil and nicardipine are increased.

Drug-drug interactions between midazolam and CCBs are due to metabolism. Metabolism of most drugs in the human body is accomplished via two reactions that occur in the liver: phase I and phase II. Phase I reactions involve the Cytochrome P-450 (CYP) enzymes located on the smooth endoplasmic reticulum of mainly hepatic and duodenal tissue. In Phase I, non-synthetic reactions the drug is chemically altered by oxidation, reduction, hydrolysis, or dealkylation or a combination of these. Many times

the products of these biotransformations are pharmacologically active metabolites.

Phase II or synthetic reactions involve the conjugation of the parent drug or its metabolite with a substrate such as an amino acid or glucuronic acid. These conjugations usually make the drug or its metabolite inactive as well as more polar and hence easier to eliminate from the body via the kidneys. CYP enzymes are the major catalysts of drug biotransformation in humans. Researchers have identified twelve cytochrome substrate families in humans. The CYP3A family is responsible for metabolism of over half of the drugs metabolized (Hardman, Limbird, Molinoff, Ruddon, & Gilman 1996).

The bulk of drug-drug interactions occur during phase I metabolism. These interactions occur when two drugs metabolized by the same CYP family compete for its metabolizing properties. The results of competition can involve toxic and or ineffective dosages of the competing drugs (Hardman et al., 1996).

Hepatic microsomal CYPs are responsible for catalyzing an extraordinary large amount of the lipophilic exogenous and endogenous compounds. CYPs accomplish this via the reactions mentioned above. When two different drugs are metabolized by the same CYP family or subfamily, a competitive atmosphere is created. This competition may slow or inhibit the metabolism of one of the drugs creating a longer than desired effect of that drug (Wrighton & Stevens, 1992). Midazolam is a drug extensively metabolized by the CYP3A subfamily. Verapamil and nicardipine also appear to be at least in part metabolized by the CYP3A family. Experiments have shown verapamil and nicardipine to have the ability to inhibit the metabolism of certain drugs by CYP3A resulting in toxic plasma concentrations (Ketter et al., 1995).

Gascon and Dayer (1991) performed an inhibition experiment with midazolam. They found verapamil and nicardipine both inhibited the in vitro metabolism of alpha hydroxymidazolam in human hepatic microsomes. However, these experiments involved the use of microsomes from only one human liver excluding the variable of inter-individual variability. The liver used in their experiment was also genetically different from the norm being a polymorphic extensive metabolizer. Dundee et al. (1986) questioned the use of polymorphic liver samples in inhibition studies, citing the possible interference of a poor metabolizer of midazolam in their study. The findings of Klotz, Mikus, Zekorn, and Eichelbaum (1986) in the metabolism of midazolam in poor and extensive metabolizers cannot exclude the possibility that genetic factors play an important role in midazolam's metabolism.

In a search of Medline between 1990 and 1998, no studies were found about the effects of midazolam's metabolism with verapamil and nicardipine in human liver microsomes in human liver samples. The need to study the effects in vitro is an important step in understanding possible in vivo interactions.

Purpose of Study

The purpose of this study was to assess the in vitro metabolic reactions of midazolam in the presence of the calcium channel blockers verapamil and nicardipine in hepatic microsomes in human livers.

Hypotheses

This quantitative, experimental study contained the following hypotheses: (a) The in vitro metabolic reactions of midazolam in the presence of verapamil in human hepatic

microsomes will be prolonged; (b) The in vitro metabolic reactions of midazolam in the presence of nicardipine in human hepatic microsomes will be prolonged.

Framework

Nursing theory is viewed as being essential to the development of nursing as a profession. Theory helps to clarify and narrow the focus of a profession enabling a clearer definition of what the profession does to enhance the lives of the public (Casey, 1996). This in vitro, experimental study was undertaken using Akinsanya's (1987) model of bionursing.

Bionursing was developed to link the biological sciences and nursing, separate from the link that exists between the life sciences and medicine. The two major assumptions which exist in the bionursing model are nurses require a knowledge of the biological sciences to be effective in practice and nursing is a task-based profession (Casey, 1996).

Akinsanya (1987) explores the notion of tasks in nursing. The bionursing model assumes four levels of tasks. The first level describes the knowledge of nursing tasks that are shared with the general public. The second level expands the knowledge base and includes things such as being able to take and record physiological observations. The third and fourth levels are much more specialized and are concerned not only with tasks such as administering medications, but possessing knowledge of the effects of drugs and courses of action to ameliorate or enhance those effects (Casey, 1996).

Akinsanya (1987) argues that if nurses perform their tasks with vigilant attention to detail then the best interests of the patients' well being and safety are brought to the forefront. The information base that gives reason to nursing actions must be clearly

understood. This information base must rely heavily on the life sciences. Nursing is more than soothing and reassuring the patient. Knowledge of biochemical environments, pharmacological interactions, and the body's responses to the disease process are some of many subjects that must be a part of the nurses' understanding. For the purpose of this study, Akinsanya's model was used to describe and test the physiological in vitro effects of verapamil and nicardipine on the metabolism of midazolam's major metabolite, alpha hydroxymidazolam. By testing the effects of these reactions concepts from Akinsanya's third and fourth levels were used.

Definition of Terms

For the purposes of this study the following terms are operationally defined.

1. Biotransformation. The conversion of xenobiotics from one form to another within an organism associated with a change in pharmacologic activity.
2. Half-life ($t_{1/2}$). The time (in hours) required for the drug concentration in the plasma to decrease by 50%.
3. High Performance Liquid Chromotography (HPLC). The separation of chemical substances by differential movement through a stationary and mobile (two-phase) system. The material to be separated is injected through a column of a chosen absorbent; the substances least absorbed emerge the soonest; those more strongly absorbed emerge later.
4. K_i. An equilibrium constant which is a measure of affinity. The effectiveness of the inhibitor is normally expressed by this constant K_i, which is the reciprocal of the enzyme inhibitor affinity. A low K_i means a high affinity and a high K_i means a low affinity.

5. Volume of distribution (Vd). The amount of drug present in the body divided by the concentration of drug in the plasma, expressed in liters.

6. Xenobiotic. A pharmacologically active substance not endogenously produced and therefore foreign to an organism.

Assumptions and Limitations

Assumptions

1. This research was being performed in vitro, so it cannot be fully determined if the same results would be observed in vivo.

2. Each sample of liver microsomes contained the same protein composition, thus yielding uniform results.

3. The stability of drug metabolizing enzymes were not altered during culture and storage.

Limitations

1. The sample size was limited by the use of microsomes from three human livers.

CHAPTER II:

REVIEW OF THE LITERATURE

The purpose of this study was to assess the *in vitro* metabolism of midazolam in the presence of verapamil and nicardipine using hepatic microsomes from three different human livers. In this chapter a theoretical and empirical review of the literature regarding midazolam and its effects with calcium channel blockers will be presented. Specifically, a background description of midazolam's pharmacokinetics and pharmacodynamics will be discussed. In addition, empirical studies regarding midazolam's effects with other drugs including CCBs will also be included.

Among its class of drugs midazolam, has properties which make it useful in anesthesia. It is a short-acting water-soluble benzodiazepine and is classified as an imidazobenzodiazepine derivative. Compare to other benzodiazepines, midazolam is unique due to its pH dependent ring structure. Midazolam's structure is such that at a pH less than 4, one of the benzene rings in the structure is open. This imparts water-solubility and negates the need for a solubilizing preparation. This property greatly reduces venous irritation and pain upon intravenous injection. At pH greater than 4 as occurs at physiological pH, the benzene ring closes and the drug becomes lipophilic. This property favors passive diffusion of the drug across cell membranes where the drug can exert its effects (Stoelting, 1991).

Pharmacokinetics

Midazolam has unique pharmacokinetic properties which when in the presence of other drugs can effect its metabolism. Midazolam is rapidly absorbed from the

gastrointestinal tract and readily crosses the blood brain barrier. A first-pass effect through the liver reduces the amount of an oral dose in circulation by approximately 50 percent. Midazolam is approximately 96% protein bound. Protein binding is independent of the plasma concentration of midazolam. The volume of distribution is 0.8 to 1.5 liter per kilogram. The volume of distribution is increased in women, the obese, and in the elderly (Dundee et al., 1984).

Intravenous midazolam metabolism occurs almost exclusively by biotransformation. The principle metabolite is alpha hydroxymidazolam (1-OH), the other 4-hydroxymidazolam (4-OH). Both metabolites are less pharmacologically active than the parent drug. The 1-OH is rapidly conjugated with glucuronic acid and makes up between 50 to 70 percent of the drug eliminated in the urine (Matsey, Panneton, Donati, & Varin, 1994).

Midazolam is metabolized by at least three different CYP enzymes. The CYP3A3, CYP3A4, and CYP3A5 isoenzymes are responsible for midazolam's biotransformations. As part of the cytochrome P450 mixed function oxidase system, metabolism by these enzymes occurs predominately in the liver. The two isoenzymes CYP3A3 and CYP3A4 metabolize midazolam to approximately the same extent. The CYP3A5 isoenzyme appears to metabolize midazolam at 2.7 times the rate of the other isoenzymes. In addition, CYP3A5 metabolism is preferential for the principal metabolite 1-OH. The isoenzymes CYP3A3 and CYP3A4 are mostly found in the liver. The isoenzyme CYP3A5 is present predominately in the kidneys. It is also found in 25 percent of human livers and in the human fetal liver, unlike the other two isoenzymes.

Anything affecting the availability of the enzymes directly influences the metabolism of midazolam (Wandel et al., 1994).

Midazolam's short duration of action is related to its lipophilicity at physiological pH. The drug's lipophilicity leads to rapid redistribution from the vessel rich groups to the inactive tissues. The clearance of midazolam has been found to be 5.8 to 9.0 ml/min/kg. The elimination half-life is from 1 to 4 hours. In comparison to the prototypical benzodiazepine diazepam, midazolam is two to three times as potent, has a shorter half-life and duration of action and a faster clearance rate (Dundee et al., 1984)

Thus, drugs such as the CCBs verapamil and nicardipine which are metabolized by the CYP3A system may inhibit midazolam's metabolism. This may in effect potentiate the effects of midazolam. The effects of midazolam on the body are found in its pharmacodynamics.

Pharmacodynamics

Midazolam produces dose-dependent respiratory depression and a decreased response to the increased partial pressure of arterial carbon dioxide. Respiratory depression is deepened with addition of opioids, alcohol, or barbituates. Midazolam decreases blood pressure but heart rate is increased by baroreceptor mediated reflexes. The baroreceptor reflex is blunted transiently. As with ventilation, central nervous system depressants will magnify cardiovascular changes. In addition, midazolam reduces cerebral blood flow and cerebral metabolic oxygen requirements, while increasing cerebral vascular resistance. It is a safe agent in patients with decreased intracranial compliance (Stoelting, 1991). Drug's inhibiting midazolam's metabolism could possibly increase or prolong the above effects.

Inhibition Studies

In addition to the studies mentioned in Chapter One, other empirical research specifically using CCBs with midazolam highlight the potential for CYP microsomal inhibition.

Hiller et al. (1990) investigated the possible influence of erythromycin on midazolam metabolism. This study was conducted after reports of unconsciousness associated with the co-administration of the two drugs to an eight-year old child. As mentioned in Chapter One this young child did have adverse effects possibly from a drug-drug interaction. Six children, three boys and three girls, with a mean age of 7 and mean weight of 28 kilograms, underwent minor otolaryngological procedures. All the children received the same dose of oral midazolam (0.5mg/kg) for sedation and the same anesthetic regimen. Plasma concentrations of midazolam were measured before the drug was given and at 30, 60, 120, 180, 240, 360, and 1440 minutes. The children were on no other drug regimen perioperatively. The mean area under the curve was 214 ng/ml/hr. In comparison, the concentration of midazolam in the child who lost consciousness was significantly greater. In his plasma evaluations the mean area under the curve over a 24-hour period was 601 ng/ml/hr.

A double-blind study by Ahonen, Olkkola, Salmenpera, Hynynen, and Neuvonen (1996) looked at the effect of the calcium channel blocker diltiazem on 30 coronary artery bypass graft patients who received midazolam and alfentanil during surgery. Patients were randomly assigned to the control group or diltiazem group prior to induction of anesthesia with midazolam and alfentanil. Those in the diltiazem group received 60 mg of diltiazem by mouth two hours prior to anesthesia induction and

maintained on a constant infusion during surgery and post-operatively for 23 hours at 0.1 mg/kg/hr. All patients were maintained on alfentanil and midazolam by constant infusion during surgery at 0.1 mg/kg/min. Elimination half-life and context-sensitive half-life were measured for midazolam and alfentanil. Discontinuance from the ventilator and tracheal extubation were also quantified between the two groups. For those who took diltiazem, the half-lives of midazolam and alfentanil were increased and these patients were extubated on average 2.5 hours later than the control group. Diltiazem and midazolam are both metabolized by the P450 CYP3A isoenzyme.

A randomized double-blind study by Backman et al. (1994) examined the effects of oral midazolam when taken in conjunction with oral CCBs verapamil and diltiazem. The study was divided into three phases. Nine healthy volunteers were administered either 80 mg of verapamil, 60 mg of diltiazem, or a placebo for three times a day over two days. On the second day the volunteers were given 15 mg of oral midazolam one hour after the last dose of CCB or placebo. Blood samples were drawn immediately before the midazolam was administered and every hour afterward for 8 hours and at 17 hours after the midazolam dose. This procedure was repeated 2 more times at 7-day intervals. Study results revealed peak concentrations and elimination half-lives of midazolam two to three times that of the placebo group. In addition psychomotor activities were decreased and sedation increased in the diltiazem and verapamil groups.

As noted in Chapter One, Gascon and Dayer (1991) conducted an inhibition study using human hepatocytes. They found the hydroxylation of midazolam to its metabolites, were inhibited in the presence of several drugs. In vitro inhibition was seen with CCBs

as well as with other classes of drugs. However, the limitation of using only one abnormal liver for microsomal preparations differs from the methodology of this study.

Ketter et al. (1995) reported that CYP3A3/4 inhibitors may increase concentrations of CYP3A3/4 substrates in the plasma and yield toxic levels. Known inhibitors of CYP3A3/4 include some antidepressants, nondihydropyridine CCBs, macrolide antibiotics, and certain antifungal preparations. Verapamil is a nondihydropyridine CCB and has been identified as a substrate and inhibitor of CYP3A3/4 enzymes.

SUMMARY

Previous studies have found that competition for CYP microsomal enzymes effects the metabolism of drugs especially the CYP3A family of enzymes which are responsible for the metabolism of midazolam, verapamil, and nicardipine. The purpose of this study was to quantify the effects of verapamil and nicardipine on the rate of metabolism of midazolam. Specifically, the formation of midazolam's major metabolite, 1-OH was investigated using hepatic microsomes from three human liver donors.

CHAPTER III:

METHODOLOGY

The experimental design of this study has been well established and previously published (Hinkle, 1997). Human microsomes were used from three donor livers. The microsomes were not pooled but kept separate, providing P450 isoenzyme samples that allow variability among the three individual liver samples. The velocity of midazolam metabolite formation was measured without interfering factors. The calcium channel blockers, verapamil and nifedipine were then added to the midazolam and the changes in midazolam metabolite velocity measured.

Microsome Extraction

The human hepatocytes were obtained from the Washington Regional Transplant Consortium in Washington D.C. They were taken from livers not appropriate for human cadaver donor transplant. The livers were determined to be suitable for scientific research. Microsomes from the cadaver liver were pooled after determining similar activity. Protein concentrations were measured using the BioRad Protein Assay, BioRad laboratories (Richmond, CA) with bovine serum albumin as the standard. Descriptive data of the donors exists detailing gender and drug history; no other means of identification is available. At the Uniformed Services University the microsomes have been stored for the past two years in refrigeration at -80°C until the time for use in research.

Chemical and Reagents

F. Hoffman-LaRoche, A.G. of Basel, Switzerland donated midazolam and 1-OH. Sigma Chemical Company of St. Louis, Missouri provided the internal standard lorazepam and the NADPH generating system. HPLC grade acetonitrile and water were purchased from Fisher Scientific of Pittsburgh, Pennsylvania, and the formic acid from the Aldrich Chemical Company (Milwaukee, Wisconsin).

Assay

To study the effect of midazolam on human liver microsomes, a baseline measurement of retention times between midazolam, 1-OH, and the internal standard lorazepam was needed. The assay developed by Hinkel (1997) using the Hewlett-Packard High Performance Liquid Chromatography System, (model 1050), was used. The absorbent column was a 75 ml column from the Phenomenex Company (Torrence, CA). The temperature of the column was set by thermostat at 35 degrees Celsius. The mobile phase solution was acetonitrile and HPLC grade water in an 80:20 ratio. The pH of Midazolam was reduced to 4.0 using 0.05% formic acid. The mobile phase pump ran at 0.3 ml/min. Injection volumes were 5 μ L with run times of 20 minutes. Flow rate gradients (time in min: flow rate ml/min) were 0:0.3, 10:0.35, 12:0.6, 14:0.6, and 15:0.3. The gradient conditions (time in min: % formic acid in acetonitrile) were 0:20, 10:55, 12:98, and 15:20.

Standard Solutions

Midazolam and 1-OH stock solutions were prepared in ethanol at a 0.32 mM concentration. Dilutions were performed for experimental conditions. A lorazepam stock solution of 3.11 mM concentration in ethanol was prepared and then diluted to the

experimental concentration of 5.0 uM. The stock solutions were stored at 5 degrees Celsius for the duration of the study.

Experimental Design

The experimental design consisted of preparation of the microsomes, incubation with midazolam and the CCBs, extraction of the metabolites from the remaining proteins, and HPLC analysis. Midazolam concentrations with nicardipine were 0.5, 2.5, and 5.0 uM. These substrate concentrations were then incubated with nicardipine concentrations of 0.5, 1.0, 5.0, and 10 uM. Due to Gascon and Dayer's study (1991) which found verapamil inhibited the formation of 1-OH in an abnormal liver (K_i of 100), the concentrations of midazolam and verapamil were increased. Substrate concentrations of midazolam were 1, 2, 6, and 12 uM. Inhibitor concentrations of verapamil were 10, 25, 50, and 100uM. Each substrate concentration was run in triplicate. This yielded approximately 135 samples plus controls and standards.

Preparation of Microsomes

After thawing at room temperature, 20 uL of microsomes were diluted with phosphate buffer to yield a final protein concentration of 0.2 mg of protein/ml. Then 10 uL of NADPH generating system was added to each microsome sample. After this each sample was warmed in a shaking water bath at 37 degrees Celsius for 3 minutes. The NADPH generating system was prepared by mixing 423 mgs of glucose 6 phosphate, 126 mgs of NADP⁺, 1230 uL of buffer, and 270 uL of glucose 6 phosphate dehydrogenase to total 1500 uL.

Incubation Phase

Ten uL of a substrate concentration of midazolam was added to each sample at zero minutes. The reaction was then incubated for 5 minutes at 37 degrees Celsius. The reaction was stopped by placing the samples in ice. Each sample differed from each other in either concentration of substrate or concentration of CCB. Two hundred uL of lorazepam (5uM) was then added to each sample and controls.

Extraction Process

Five mls of acetonitrile was added and the test tubes vortexed for 10 minutes. The test tubes were centrifuged at 2000g and 5 degrees Celsius for 10 minutes. The samples were transferred to clean tubes and labeled. Following this, the samples were evaporated with a speed vacuum apparatus. Twenty uL of acetonitrile and water in a 1:1 ratio was added and the samples vortexed for 3 minutes. Two mls of acetonitrile were then added and again vortexed for 3 minutes and centrifuged at 2000g and 5 degrees Celsius for 10 minutes. Once again the contents were transferred to clean tubes, labeled and evaporated with the vacuum apparatus. After evaporation, 20 uL of acetonitrile and water in a 1:1 ratio was added and each test tube vortexed for 2 minutes. Following this 20 uL of water was added. The test tubes were then vortexed for 2 minutes and transferred to microvials and loaded onto the HPLC system.

HPLC Analysis

The peaks of midazolam, 1-OH, and lorazepam were visualized. Integration (isolating the correct peaks by computer manipulation) was performed if retention times were different from known velocities of midazolam and 1-OH.

The K_i and percent of inhibition for 1-OH was measured by non-linear regression. Regression analysis measures the averages between points and generates a curve that follows an average path. Determining the K_i required graphing $1/\text{velocity}$ versus $1/\text{midazolam concentration}$ to determine the slope of each line. Then the slope versus the concentration of the CCB was plotted. The x-intercept of the line is the K_i .

CHAPTER IV:

ANALYSIS OF DATA

We investigated the *in vitro* metabolic effects of the CCBs verapamil and nicardipine on the metabolism of midazolam. Of principle interest was the formation of 1-OH, midazolam's major metabolite. Human cadaver liver microsomes from three livers were used. The microsomes were kept separate to allow for interindividual variation. Substrate, inhibitor, and enzyme generating system concentrations were added to the liver microsomes. The samples were then incubated, the protein extracted and then loaded onto the HPLC system to measure the formation of 1-OH.

Hypotheses

This quantitative experimental study postulated the following hypotheses:

1. The *in vitro* metabolic reactions of midazolam in the presence of verapamil in human hepatic microsomes will be prolonged.
2. The *in vitro* metabolic reactions of midazolam in the presence of nicardipine in human hepatic microsomes will be prolonged.

Verapamil and nicardipine inhibited the formation of midazolam's principle metabolite 1-OH in human liver microsomes. These CCBs had differential effects on midazolam metabolism. The rank order of potency against the formation of 1-OH was nicardipine > verapamil. Nicardipine was a potent inhibitor of midazolam with respective apparent K_i values of 1.2, 0.8, and 2.05 μM for microsomes 16, 18, and 12 (Figure 1). The velocity of 1-OH formation was reduced in the presence of nicardipine at each concentration of substrate and inhibitor (Table 1). The formation

of 1-OH as a percent of control was also reduced in the presence of nicardipine at each concentration of substrate and inhibitor (Table 2).

Verapamil showed apparent less potency than nicardipine in inhibiting 1-OH formation. Respective K_i values of 24, 27, and 37 μM for microsomes 16, 18, and 13 (Figure 1). The velocity of formation of 1-OH was reduced at each concentration of substrate and inhibitor (Table 3). In addition, the formation of 1-OH as a percent of control was reduced in each concentration of midazolam and verapamil (Table 4).

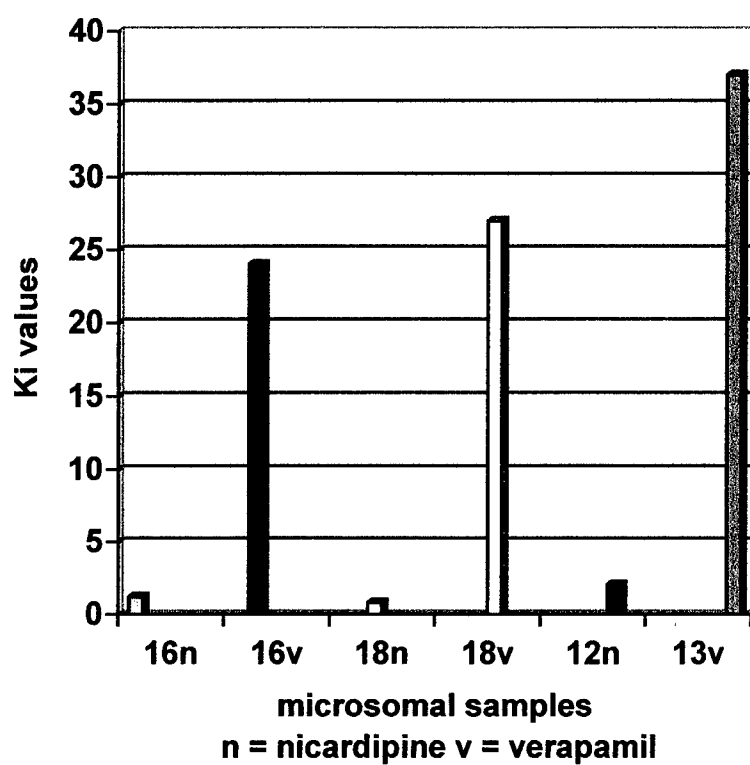


Figure 1.

Ki values for microsomes 16,18,12, and 13.

Table 1.**Velocity of 1-OH Formation**

Nicardipine						
mcs#16						
<u>nicardipine uM</u>		0	0.5	1.0	5.0	10
		1-OH uM				
<u>mdz uM</u>	0.5	0.22	0.19	0.14	0.02	-
	2.5	1.16	0.88	0.62	0.37	0.19
	5.0	2.24	1.26	1.10	0.68	0.51
mcs#18						
	0.5	0.14	0.07	0.09	0.01	0.009
	2.5	0.52	0.34	0.33	0.22	0.21
	5.0	0.79	0.58	0.46	0.36	0.34
mcs#12						
	0.5	0.02	0.017	0.012	0.011	-
	2.5	0.064	0.028	0.027	0.02	-
	5.0	0.071	0.04	0.033	0.029	0.014

Note. Values bolded represent the velocity of 1-OH formation. uM = micromolar concentration. mcs# = corresponding microsomal sample from numbered liver donor; mdz = midazolam. Dashes indicate the velocity of formation was not obtained.

Table 2.**1-OH Formation as a Percent of Control**

Nicardipine

Nicardipine uM		0.5	1	5	10
mcs#16					
1-OH uM	0.5	86.36	63.64	9.09	-
	2.5	75.86	53.45	31.89	16.38
	5.0	56.25	49.11	30.36	22.77
mcs#18					
	0.5	50	64.29	7.14	6.43
	2.5	65.38	63.46	42.31	40.38
	5.0	73.41	58.23	45.57	43.04
mcs#12					
	0.5	85	60	55	-
	2.5	43.75	42.19	31.25	-
	5.0	56.34	46.48	40.85	19.72

Note. Bolded numbers represent formation of 1-OH as a percent of control. mcs# = corresponding microsomal sample from numbered liver donor; uM = micromolar concentraion.

Table 3.**Velocity of 1-OH Formation**

Verapamil

mcs#16						
<u>Verapamil uM</u>		0	10	25	50	100
		1-OH uM				
<u>mdz uM</u>						
1		0.76	0.32	0.37	0.27	0.28
2		1.32	0.65	0.63	0.51	0.41
6		2.96	1.60	1.50	1.03	0.60
12		3.60	2.90	2.30	1.60	0.99
mcs#18						
1		0.46	0.26	0.19	0.13	0.12
2		0.66	0.42	0.35	0.23	0.15
6		1.12	0.89	0.65	0.54	0.30
12		1.58	1.17	0.92	0.81	0.55
mcs#13						
1		0.41	0.24	0.18	0.14	0.12
2		0.60	0.42	0.28	0.23	0.08
6		1.31	0.80	0.72	0.43	0.05
12		1.41	1.10	0.89	0.71	0.16

Note. Values bolded represent the velocity of 1-OH formation. uM = micromolar concentration. mcs# = corresponding microsomal sample from numbered liver donor; mdz = midazolam.

Table 4.**1-OH formation as a percent of control****Verapamil**

Verapamil uM					
mcs#16		10	25	50	100
1-OH uM	1	42.10	48.68	35.52	36.84
	2	49.20	47.72	38.63	31.06
	6	54.05	50.67	34.79	20.27
	12	80.55	63.88	44.44	27.50
mcs#18					
	1	56.52	41.30	28.26	26.08
	2	63.63	53.03	34.84	22.72
	6	79.46	58.03	48.21	26.78
	12	74.05	58.22	51.26	34.81
mcs#13					
	1	58.53	43.90	34.14	29.26
	2	70.00	46.66	38.33	13.30
	6	61.06	54.96	32.82	3.81
	12	78.01	63.12	50.35	11.34

Note. Bolded numbers represent formation of 1-OH as a percent of control. mcs# = corresponding microsomal sample from numbered liver donor; uM = micromolar concentration.

CHAPTER V:

SUMMARY

Nurse anesthetists give several drugs to patients during the administration of anesthesia. These patients are often already on a variety of medications. Competition for cytochrome P450 enzymes affects the metabolism of drugs to varying degrees. The CYP3A family of enzymes are responsible for the metabolism of midazolam, nicardipine, and verapamil. The effects of nicardipine and verapamil were quantified on the metabolism of midazolam, specifically the formation of midazolam's principal metabolite 1-OH.

Microsomes were used from three human cadaver donor livers. The microsomes were not pooled together but kept separate during testing procedures. Experimental design involved preparation of microsomes, incubation with midazolam and the CCBs, extraction of metabolites, and finally HPLC analysis.

Conclusions

Inhibition of midazolam's principal metabolite 1-OH, was seen in the presence of both CCBs. The degree of inhibition differed between nicardipine and verapamil. Nicardipine showed stronger inhibitory effects than verapamil. The mean K_i value for nicardipine was 1.35 μM and for verapamil 29.3 μM . The lower the K_i value the stronger the inhibitor. As CCB concentration increased and substrate midazolam was held constant the velocity of 1-OH formation decreased.

Recommendations for Future Practice

These findings suggest that nicardipine and verapamil will prolong the metabolism of midazolam and potentially increase the pharmacodynamic effects of this

drug. This knowledge is important for the nurse anesthetist. According to Akinsanya's (1987) framework of bionursing the nurse must have knowledge of the biological sciences to be effective in their practice. Possessing the knowledge of pharmacological effects and their interactions is the highest level of Akinsanya's framework. Nurses should be knowledgeable of the medications they administer, their possible drug-drug interactions, and the steps to enhance desired effects or ameliorate unwanted side effects and interactions. Continued study in pharmacology, physiology, and co-existing diseases are some ways that the nurse can gain the knowledge to reach Akinsanya's highest level of nursing and provide safe, professional care for their patients.

Recommendations for Future Studies

When predicting in vivo drug interactions from in vitro metabolic inhibition data, other factors should be considered. The degree of unbound drug in plasma, enzyme induction, and drug concentration at the site of metabolic activity, are variables warranting further investigation. Larger sample sizes and variations in race, gender, and age also need consideration when investigating the possible drug-drug interactions between midazolam and the calcium channel blockers.

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